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Effect of *Rj* Genotype and Cultivation Temperature on the Community Structure of Soybean-Nodulating Bradyrhizobia

Sokichi Shiro,[a] Akihiro Yamamoto,[b] Yosuke Umehara,[c] Masaki Hayashi,[c] Naoto Yoshida,[b] Aya Nishiwaki,[b] Takeo Yamakawa,[d] and Yuichi Saeki[b]

Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, Miyazaki, Japan[a]; Faculty of Agriculture, University of Miyazaki, Miyazaki, Japan[b]; National Institute of Agrobiological Sciences, Ibaraki, Japan[c]; and Faculty of Agriculture, Kyushu University, Fukuoka, Japan[d]

The nodulation tendency and community structure of indigenous bradyrhizobia on *Rj* genotype soybean cultivars at cultivation temperatures of 33/28°C, 28/23°C, and 23/18°C for 16/8 h (day/night degrees, hours) were investigated using 780 bradyrhizobial DNA samples from an Andosol with 13 soybean cultivars of four *Rj* genotypes (non-*Rj*, Rj_2Rj_3, Rj_4, and $Rj_2Rj_3Rj_4$). A dendrogram was constructed based on restriction fragment length polymorphism of the PCR products (PCR-RFLP) of the 16S-23S rRNA gene internal transcribed spacer region. Eleven *Bradyrhizobium* U.S. Department of Agriculture strains were used as a reference. The dendrogram indicated seven clusters based on similarities among the reference strains. The occupancy rate of the Bj123 cluster decreased with increasing cultivation temperature, whereas the occupancy rates of the Bj110 cluster, Be76 cluster, and Be94 cluster increased with increasing cultivation temperature. In particular, the $Rj_2Rj_3Rj_4$ genotype soybeans were infected with a number of Bj110 clusters, regardless of the increasing cultivation temperature, compared to other *Rj* genotype soybean cultivars. The ratio of beta diversity to gamma diversity (H'_β/H'_γ), which represents differences in the bradyrhizobial communities by pairwise comparison among cultivation temperature sets within the same soybean cultivar, indicated that the bradyrhizobial communities tended to be different among cultivation temperatures. Multidimensional scaling analysis indicated that the infection of the Bj110 cluster and the Bj123 cluster by host soybean genotype and the cultivation temperature affected the bradyrhizobial communities. These results suggested that the *Rj* genotypes and cultivation temperatures affected the nodulation tendency and community structures of soybean-nodulating bradyrhizobia.

Soybean [*Glycine max* (L.) Merr.] forms root nodules by infection with the soybean-nodulating bacteria bradyrhizobia and acquires atmospheric nitrogen as ammonia through the root nodules. In soybean cultivation, inoculation of the bradyrhizobia that show a high ability for nitrogen fixation may increase the soybean yield, but the efficiency of inoculum with high nitrogen fixation ability is poor in soybean fields because the inoculum cannot compete with indigenous bradyrhizobia in the soil. To solve this problem, it is very important to understand the ecology of indigenous bradyrhizobia in terms of the genetic diversity, geographical distribution, compatibility with the host soybean, and environmental factors associated with localization and dominance in the soil.

Saeki et al. (21) investigated the genetic diversity and geographical distribution of indigenous bradyrhizobia isolated from five sites in Japan (Hokkaido, Fukushima, Kyoto, Miyazaki, Okinawa) by PCR restriction fragment length polymorphism (PCR-RFLP) analysis of the 16S-23S rRNA gene internal transcribed spacer (ITS) region and revealed that geographical distribution of indigenous bradyrhizobia varied from the northern to southern regions in Japan. As a result, the representative clusters of isolated indigenous bradyrhizobia were in the order of *Bradyrhizobium japonicum* USDA 123, 110, and 6 and *Bradyrhizobium elkanii* USDA 76^T clusters from northern to southern regions in Japan. It has been suggested that an environmental factor such as temperature will influence the localization of Japanese indigenous bradyrhizobia. Saeki et al. (23) investigated the occupancy of three *Bradyrhizobium japonicum* strains and one *Bradyrhizobium elkanii* strain under different temperature conditions in soil and liquid media and suggested that temperature is one of the environmental factors that affects the occupancy of indigenous bradyrhizobia in soil.

The *Rj* genes are known as nodulation regulatory genes, and the *Rj* genotypes of non-*Rj*, rj_1, Rj_2, Rj_3, and Rj_4 have been confirmed to exist naturally (4, 29, 30, 32). The *Rj* genotype soybean cultivars and variety of bradyrhizobial strains were investigated for compatibility and their preference for nodulation (8). The $Rj_2Rj_3Rj_4$ genotype lines were bred by crossing the IAC-2 (Rj_2Rj_3) and Hill (Rj_4) cultivars (9). Yamakawa et al. (33) investigated the preference for nodulation of *Bradyrhizobium japonicum* to the $Rj_2Rj_3Rj_4$ genotype lines and demonstrated that $Rj_2Rj_3Rj_4$ genotype lines are superior to non-*Rj*, Rj_2Rj_3, and Rj_4 genotypes for the formation of efficient nodules for inoculum with high nitrogen fixation ability. The utilization of the $Rj_2Rj_3Rj_4$ genotype lines soybean might therefore increase the soybean yield. Minami et al. (14) isolated 260 indigenous bradyrhizobia from 13 soybean cultivars of five *Rj* genotypes (non-*Rj*, Rj_2Rj_3, Rj_3, Rj_4, and $Rj_2Rj_3Rj_4$) from an Andosol and estimated the nodulation tendency among *Rj* genotype soybeans. The results showed that indigenous bradyrhizobial communities among the same *Rj* genotype soybean cultivars were similar to each other, whereas indigenous bradyrhizobial communities between the Rj_2Rj_3 genotype and non-*Rj*, Rj_3, or Rj_4 genotype soybean cultivars were significantly different. However, the nodulation tendency of indigenous bradyrhizobia under

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Address correspondence to Yuichi Saeki, yt-saeki@cc.miyazaki-u.ac.jp.

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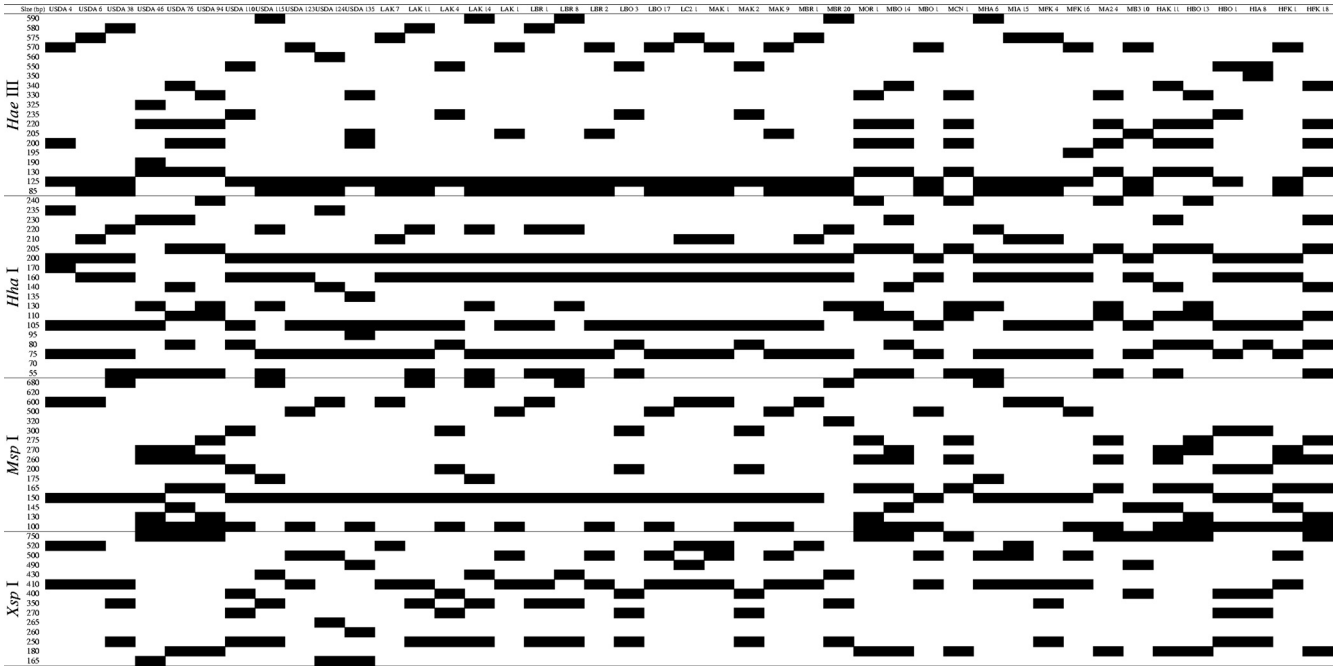

FIG 1 Schematic representation of the RFLP patterns of the 16S-23S rRNA gene ITS region. Reference strains and representative isolates are indicated as follows: USDA 4, 6, 38, 110, 115, 123, 124, and 135 represent *Bradyrhizobium japonicum* USDA 4, 6^T, 38, 110, 115, 123, 124, and 135, respectively, and USDA 46, 76, and 94 represent *Bradyrhizobium elkanii* USDA 46, 76^T, and 94, respectively. Low: Akishirome, Bragg, Bonminori, and C242 are represented by LAK, LBR, LBO, and LC2 respectively. Middle: Akishirome, Bragg, Orihime, Bonminori, CNS, Hardee, IAC-2, Fukuyutaka, A-250-3, and B349 are represented by MAK, MBR, MOR, MBO, MCN, MHA, MIA, MFK, MA2, and MB3, respectively. High: Akishirome, Bonminori, IAC-2, and Fukuyutaka are represented by HAK, HBO, HIA, HFK, respectively.

different cultivation temperature conditions was not investigated in that study.

In the present study, to examine the influence of combinations of several cultivation temperatures and *Rj* genotype soybean cultivars on the nodulation tendency and community structure of indigenous bradyrhizobia, we isolated indigenous bradyrhizobia from an Andosol using soybean cultivars of different *Rj* genotypes and several cultivation temperatures. The isolates were analyzed by PCR-RFLP of the 16S-23S rRNA gene ITS region, and a dendrogram was constructed to classify the isolates into clusters. The effects of cultivation temperature and *Rj* genotype on soybean-nodulating bradyrhizobial communities were also estimated.

MATERIALS AND METHODS

Soybean cultivars and soil samples. We used 13 soybean cultivars of four *Rj* genotypes to investigate the effect of several cultivation temperatures and *Rj* genotypes of host soybean cultivars. The soybean cultivars were Akishirome (AK), Bragg (BR) and Orihime (OR) as non-*Rj* genotypes, Bonminori (BO), CNS (CN), Hardee (HA) and IAC-2 (IA) as Rj_2Rj_3 genotypes, Akisengoku (AG), Fukuyutaka (FK) and Hill (HI) as Rj_4 genotypes, and A-250-3 (A2), B349 (B3), and C242 (C2) as $Rj_2Rj_3Rj_4$ genotypes (9, 33). As the soil sample, an Andosol (pH [H_2O] 5.04, electrical conductivity [EC] = 0.05 dS m^{-1}; The National Agricultural Research Center for the Tohoku Region, Arai, Fukushima, Japan [14, 21]) was used for soybean cultivation because a high diversity of indigenous bradyrhizobia has been found in this soil in previous studies (21, 22).

Soybean cultivation. To isolate indigenous bradyrhizobia, we grew soybean cultivars in 1-liter culture pots for 4 weeks. The culture pots were filled with vermiculite with N-free nutrient solution (18) at 40% (vol/vol) water content and then autoclaved at 121°C for 20 min. Soybean seeds were sterilized by soaking them in 70% ethanol for 30 s and in a dilute sodium hypochlorite solution (0.25% available chlorine) for 3 min and then washing them with sterile distilled water. A soil sample (2 to 3 g) was placed in the vermiculite at a depth of 2 to 3 cm, and the soybean seeds were sown on the soil. The plants were grown for 4 weeks in a growth chamber (low: day, 23°C for 16 h, and night, 18°C for 8 h; middle: day, 28°C for 16 h, and night, 23°C for 8 h; and high: day, 33°C for 16 h, and night, 28°C for 8 h) with a weekly supply of sterile distilled water. After cultivating, 20 nodules were randomly collected from among all of the nodules harvested from soybean roots and sterilized by soaking them in 70% ethanol for 3 min and in a diluted sodium hypochlorite solution (0.25% available chlorine) for 30 min and then washing them with sterile distilled water.

DNA samples of indigenous bradyrhizobia. Total DNA for the PCR template was extracted from a nodule directly as described by Hiraishi et al. (7) with slight modifications (18). Each nodule was homogenized in 50 μl of BL buffer (40 mM Tris-HCl, 1% Tween 20, 0.5% Nonidet P-40 and 1 mmol of EDTA $liter^{-1}$ [pH 8.0]), 40 μl of sterile distilled water, and 10 μl of proteinase K (1 mg ml^{-1}) and then incubated at 60°C for 20 min and 95°C for 5 min. After centrifugation, the supernatant was collected and used as the PCR template. The DNA samples were numbered as follows: LAK 1-20, LBR 1-20, LOR 1-20, LBO 1-20, LCN 1-20, LHA 1-20, LIA 1-20, LAG 1-20, LFK 1-20, LHI 1-20, LA2 1-20, and LB3 1-20, LC2 1-20 (low cultivation temperature: Akishirome, Bragg, Orihime, Bonminori, CNS, Hardee, IAC-2, Akisengoku, Fukuyutaka, Hill, A-250-3, B349, and C242); MAK 1-20, MBR 1-20, MOR 1-20, MBO 1-20, MCN 1-20, MHA 1-20, MIA 1-20, MAG 1-20, MFK 1-20, MHI 1-20, MA2 1-20, MB3 1-20, and MC2 1-20 (middle cultivation temperature: Akishirome, Bragg, Orihime, Bonminori, CNS, Hardee, IAC-2, Akisengoku, Fukuyutaka, Hill, A-250-3, B349, and C242); and HAK 1-20, HBR 1-20, HOR 1-20, HBO 1-20, HCN 1-20, HHA 1-20, HIA 1-20, HAG 1-20, HFK 1-20, HHI 1-20, HA2 1-20, HB3 1-20, and HC2 1-20 (high cultivation temperature: Akishirome, Bragg, Orihime, Bonminori, CNS, Hardee, IAC-2, Akisengoku,

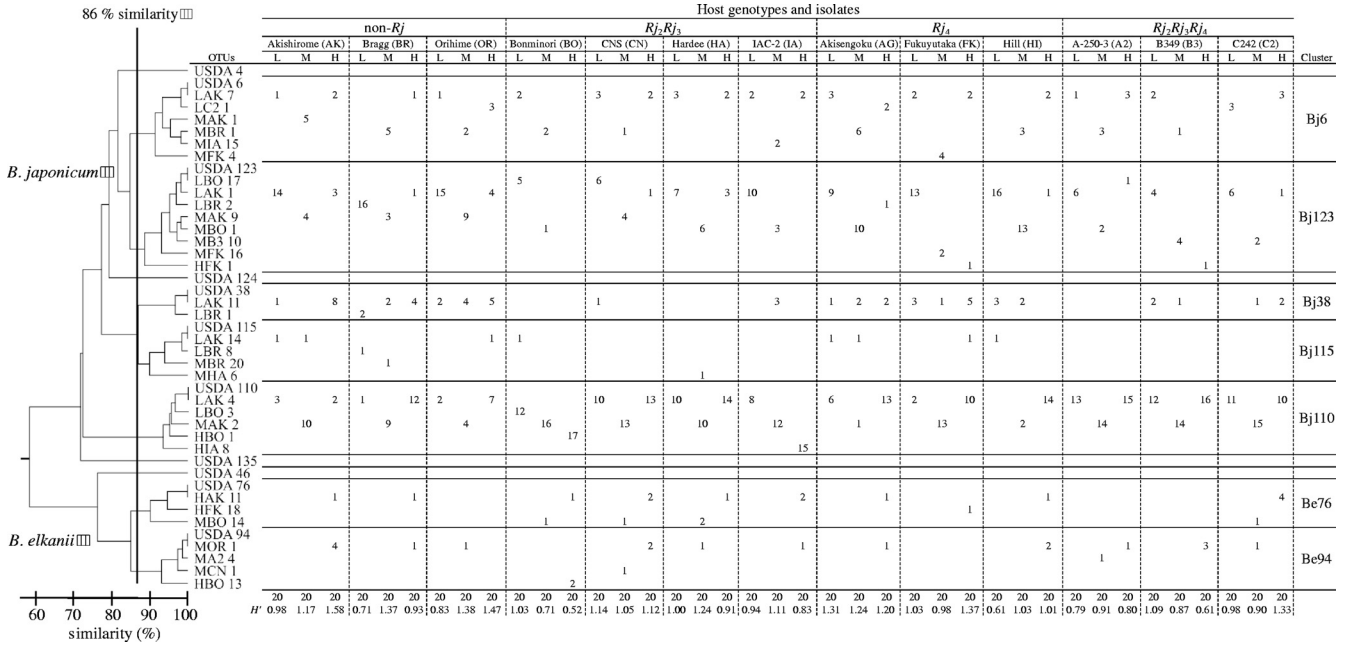

FIG 2 Dendrogram of the 16S-23S rRNA gene ITS region of indigenous bradyrhizobial DNA samples at low, middle, and high cultivation temperatures and *Bradyrhizobium* USDA reference strains. The similarity between *Bradyrhizobium japonicum* USDA 38 and 115 (which was 86%) was applied as the criterion to differentiate the clusters. Clusters were designated as indicated on the right. Tabulated numbers indicate the number of isolates in operational taxonomic units (OUTs) in all host soybean cultivars. The names of OTUs, representative isolates, were expressed in all cultivation temperature-host soybean cultivar combinations. Low, middle, and high cultivation temperatures are indicated by L, M, and H, respectively. The diversity index (H') was calculated by using the equation $H' = -\Sigma\, Pi \ln Pi$.

Fukuyutaka, Hill, A-250-3, B349, and C242). A total of 780 DNA samples were obtained for further analysis.

PCR-RFLP analysis of the 16S-23S rRNA gene ITS region. As reference strains, total DNA for PCR template of strains *Bradyrhizobium japonicum* USDA 4, 6^T, 38, 110, 115, 123, 124, and 135 and *Bradyrhizobium elkanii* USDA 46, 76^T, and 94 (19) was prepared as described previously (19). PCR was carried out using *Ex Taq* DNA polymerase (TaKaRa Bio, Otsu, Japan). For ITS amplification, we used the ITS primer set BraITS-F (5′-GACTGGGGTGAAGTCGTAAC-3′) and BraITS-R (5′-ACGTCCTT CATCGCCTC-3′) (21). The PCR cycle consisted of a pre-run at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. This temperature control sequence was repeated for a total of 30 cycles and was followed by a final post-run at 72°C for 10 min. The RFLP analysis of the 16S-23S rRNA gene ITS region was investigated using restriction enzymes HaeIII, HhaI, MspI, and XspI (TaKaRa Bio) (19). A 5-μl aliquot of the PCR product was digested with restriction enzyme at 37°C for 16 h in a 20-μl reaction mixture. The restricted fragments were separated by agarose gel electrophoresis and visualized with ethidium bromide.

Cluster analysis. For the cluster analysis, we calculated the genetic distance between pairs of isolates (D) using the following equation:

$$D = 1 - [2N_{AB}/(N_A + N_B)]$$

where N_{AB} represents the number of RFLP bands shared by the two strains, and N_A and N_B represent the numbers of RFLP bands in strains A and B, respectively (15, 24). The cluster analysis was carried out using the unweighted pair group method with arithmetic average (UPGMA) method. The dendrograms were constructed using the PHYLIP software program v3.69 (J. Felsenstein, University of Washington, Seattle, WA).

Diversity analysis of bradyrhizobial communities. To estimate the diversity of the bradyrhizobial communities isolated from the host soybean cultivars, we used the Shannon-Wiener diversity index (12, 17, 22). The formula for the diversity index was

$$H' = -\Sigma\, Pi \ln Pi$$

where Pi is the dominance of the isolates expressed by ni/N, N is the total number of tested isolates ($n = 20$), and ni is the total number of tested isolates belonging to a particular dendrogram cluster. The indexes of alpha diversity (H'_α), beta diversity (H'_β), and gamma diversity (H'_γ) were calculated (10, 31). These diversity indices were used to estimate the differences in the bradyrhizobial communities between cultivation temperature pairs. The H'_α index represents a weighted average of the diversity indices of each of the two bradyrhizobial communities, the H'_β index represents the differences between the two bradyrhizobial communities from the two host soybean cultivars and the H'_γ index represents the diversity of the total isolate communities from the two host soybean cultivars ($n = 40$). The relationship among these indices is

$$H'_\beta = H'_\gamma - H'_\alpha$$

We also estimated the differences among the compositions of the bradyrhizobial communities by comparing the ratio of the beta to the gamma index (H'_β/H'_γ), taking into consideration the difference in gamma diversity in each pairwise comparison of bradyrhizobial communities.

MDS methods and cluster analysis. To estimate the characteristics of bradyrhizobial communities in the infections of host soybeans, we performed multidimensional scaling (MDS) analysis based on Bray-Curtis similarity measures. The Bray-Curtis similarity measure was found to have not only a robust monotonic relationship with ecological distance but also a robust linear relationship with ecological distance until it became large. Because of this, Bray-Curtis similarity measure was estimated to be the index that best reflected the properties between communities (6). The Bray-Curtis similarity measure was calculated using the following equation:

$$d_{XY} = \Sigma|n_X - n_Y|/(N_X + N_Y)$$

where d_{XY} is the dissimilarity between communities X and Y, and n_X and

TABLE 1 Nodule occupancy rate of soybean-nodulating bradyrhizobia for cluster analysis[a]

	Mean nodule occupancy (%) ± SE of clusters on each *Rj* genotype											
	Non-*Rj*			*Rj*$_2$*Rj*$_3$			*Rj*$_4$			*Rj*$_2$*Rj*$_3$*Rj*$_4$		
Cluster	Low	Middle	High	Low	Middle	High	Low	Middle	High	Low	Middle	High
Bj6	3.3 ± 1.7^{B}	20.0 ± 5.0^{A}	10.0 ± 2.9^{AB}	12.5 ± 1.4	6.3 ± 2.4	7.5 ± 2.5	8.3 ± 4.4	21.7 ± 4.4	10.0 ± 5.0	10.0 ± 2.9	6.7 ± 4.4	10.0 ± 5.0
Bj38	8.3 ± 1.7	10.0 ± 5.8	28.3 ± 6.0	1.25 ± 1.3	3.8 ± 3.8	–	11.7 ± 3.3	8.3 ± 1.7	3.3 ± 3.3	3.3 ± 3.3	3.3 ± 1.7	3.3 ± 3.3
Bj110	10.0 ± 2.9	38.3 ± 9.3	35.0 ± 14.4	50.0 ± 4.1^{B}	63.8 ± 6.3^{AB}	73.8 ± 4.3^{A}	13.3 ± 8.8	26.7 ± 19.2	68.3 ± 9.3	60.0 ± 2.9	71.7 ± 1.7	68.3 ± 9.3
Bj115	3.3 ± 1.7	3.3 ± 1.7	1.7 ± 1.7	1.25 ± 1.3	1.3 ± 1.3	–	3.3 ± 1.7	1.7 ± 1.7	–	–	–	–
Bj123	75.0 ± 2.9^{A}	26.7 ± 9.3^{B}	13.3 ± 4.4^{C}	35.0 ± 5.4^{A}	17.5 ± 5.2^{AB}	5.0 ± 3.5^{B}	63.3 ± 10.1^{A}	41.7 ± 16.4^{AB}	5.0 ± 0.0^{B}	26.7 ± 3.3^{A}	13.3 ± 3.3^{B}	5.0 ± 0.0^{B}
Be76	–	–	3.3 ± 1.7	–	5.0 ± 2.0	7.5 ± 1.4	–	–	5.0 ± 0.0	–	1.7 ± 1.7	6.7 ± 6.7
Be94	–	1.7 ± 1.7	8.3 ± 6.0	–	2.5 ± 1.4	6.3 ± 2.4	–	–	5.0 ± 2.9	–	3.3 ± 1.7	6.7 ± 4.4

[a] The nodule occupancy rate was expressed as the mean ($n = 3$ or 4) of DNA samples belonging to certain clusters within identical *Rj* genotypes. Significantly different test results were found for each *Rj* genotype. Different superscript capital letters indicate significant differences (Tukey-Kramer test) at $P < 0.05$ for different cultivation temperatures in the same *Rj* genotype. –, not detected.

n_Y represent the number of strains in same cluster of X and Y, and N_X and N_Y represent the total number of strains in X and Y, respectively (3, 13). MDS analysis and the cluster analysis based on the Bray-Curtis similarity measure were conducted using the R software program v2.12.1 (The R Project for Statistical Computing: http://www.r-project.org/; University of Tsukuba, Japan: http://cran.md.tsukuba.ac.jp/). Also, the cluster analysis was carried out using the UPGMA method.

RESULTS

PCR-RFLP analysis of the 16S-23S rRNA gene ITS region. The PCR products of amplified 16S-23S rRNA gene ITS region were digested by four restriction enzymes, and the restriction fragments were separated by electrophoresis. A schematic representation of the restriction fragment patterns of the experimental plot is shown in Fig. 1. The fragment sizes were estimated using a 50-bp ladder marker. A total of 36 operational taxonomic units (OTUs) containing 11 reference strains were detected (Fig. 1). The results of phylogenetic analysis are shown in Fig. 2. The dendrogram was generated using the differences in fragment size and pattern. The maximum similarity among OTUs of the reference strains was 86% and occurred between *B. japonicum* USDA 38 and 115. These results were then applied as the criterion for distinguishing clusters in the dendrogram, which produced 11 clusters, each of which contained 11 reference strains. The indigenous bradyrhizobia isolates in the middle and high cultivation temperatures were classified into seven clusters, Bj6, Bj38, Bj110, Bj115, Bj123, Be76, and Be94, while the indigenous bradyrhizobia isolates in the low cultivation temperature were classified into five clusters, Bj6, Bj38, Bj110, Bj115, and Bj123 (Fig. 2). For the low and middle cultivation temperatures, most of the indigenous bradyrhizobia were classified into four major clusters, Bj6, Bj38, Bj110, and Bj123, while most of the indigenous bradyrhizobia in the high cultivation temperature were classified into five major clusters, Bj6, Bj38, Bj110, Be76, and Be94. The indigenous bradyrhizobia belonging to the Bj123 cluster was not a major cluster at the high cultivation temperature (Fig. 2).

Cluster occupancy of each *Rj* genotype and cultivation temperature. Cluster analysis provided us with information about the cluster occupancy of each *Rj* genotype and cultivation temperature. The occupancy rate of the Bj6, Bj38, Bj110, Bj115, Bj123, Be76, and Be94 clusters on the non-*Rj*, *Rj*$_2$*Rj*$_3$, *Rj*$_4$, and *Rj*$_2$*Rj*$_3$*Rj*$_4$ genotype soybean cultivars is shown in Table 1. Interestingly, the occupancy rate of Bj123 cluster was significantly decreased with increasing cultivation temperature. On the other hand, the occupancy rate of Bj110 cluster tended to increase with increasing cultivation temperatures. The Be76 and Be94 clusters had the same tendency as Bj110 cluster, but their occupancy rates were lower than that of Bj110 cluster (Table 1).

Diversity analysis of the bradyrhizobial communities at various cultivation temperatures. The differences in bradyrhizobial communities between the cultivation temperatures among the soybean cultivars were estimated based on the H'_β. The values of H'_β of five soybean cultivars, including the non-*Rj* and *Rj*$_4$ genotypes Akishirome, Bragg, Akisengoku, Fukuyutaka, and Hill, showed that the bradyrhizobial communities have a tendency to change greatly in response to the cultivation temperature (Tables 2 and 3). Among them, the values of H'_β of Bragg and Hill showed a larger difference in bradyrhizobial communities between the low-middle and middle-high cultivation temperatures pairs (Tables 2 and 3). On the other hand, five soybean cultivars with the *Rj*$_2$*Rj*$_3$ genotype—CNS, Hardee, A-250-3, B349, and C242—showed a tendency for the bradyrhizobial communities to change very little in response to the cultivation temperatures (Tables 2 and 3). The differences in bradyrhizobial communities among the cultivation temperatures of each *Rj* genotype were also estimated

TABLE 2 Alpha, beta, and gamma diversity indices for each cultivation temperature pair for each host soybean cultivar: non-*Rj* and *Rj*$_2$*Rj*$_3$ genotypes[a]

	Diversity for each cultivation temp pair for each host soybean cultivar																				
	Non-*Rj*									*Rj*$_2$*Rj*$_3$											
	Akishirome			Bragg			Orihime			Bonminori			CNS			Hardee			IAC-2		
Index	L-M	L-H	M-H	L-M	L-H	M-H	L-M	L-H	M-H	L-M	L-H	M-H	L-M	L-H	M-H	L-M	L-H	M-H	L-M	L-H	M-H
α diversity ($H'\alpha$)	1.07	1.28	1.37	1.04	0.82	1.15	1.10	1.15	1.43	0.87	0.78	0.61	1.10	1.13	1.09	1.12	0.96	1.08	1.02	0.88	0.97
β diversity ($H'\beta$)	0.18	0.28	0.33	0.30	0.56	0.27	0.06	0.17	0.07	0.08	0.20	0.09	0.08	0.14	0.04	0.12	0.05	0.09	0.11	0.25	0.16
γ diversity ($H'\gamma$)	1.25	1.57	1.71	1.34	1.38	1.41	1.16	1.32	1.50	0.95	0.98	0.70	1.17	1.27	1.12	1.24	1.01	1.17	1.14	1.14	1.13

[a] L-M, L-H, and M-H indicate combinations of each cultivation temperatures: low and middle, low and high, and middle and high, respectively.

TABLE 3 Alpha, beta, and gamma diversity indices for each cultivation temperature pair for each host soybean cultivar: Rj_4 and $Rj_2Rj_3Rj_4$ genotypes[a]

	Diversity for each cultivation temp pair for each host soybean cultivar																	
	Rj_4									$Rj_2Rj_3Rj_4$								
	Akisengoku			Fukuyutaka			Hill			A-250-3			B349			C242		
Index	L-M	L-H	M-H	L-M	L-H	M-H	L-M	L-H	M-H	L-M	L-H	M-H	L-M	L-H	M-H	L-M	L-H	M-H
α diversity ($H'\alpha$)	1.27	1.25	1.21	1.00	1.20	1.18	0.82	0.81	1.02	0.85	0.80	0.86	0.98	0.85	0.74	0.93	1.15	1.11
β diversity ($H'\beta$)	0.07	0.18	0.34	0.25	0.27	0.09	0.11	0.60	0.37	0.06	0.08	0.005	0.01	0.15	0.11	0.14	0.15	0.11
γ diversity ($H'\gamma$)	1.34	1.43	1.55	1.25	1.47	1.27	0.93	1.41	1.39	0.91	0.88	0.86	0.99	1.00	0.85	1.07	1.31	1.23

[a] L-M, L-H, and M-H indicate combinations of each cultivation temperatures: low and middle, low and high, and middle and high, respectively.

by the H'_β/H'_γ ratios. There was no significant difference (Tukey-Kramer test) based on the cultivation temperature in each *Rj* genotype due to the variation of bradyrhizobial communities among each *Rj* genotype soybean cultivars was large, but the values of H'_β/H'_γ between low and high cultivation temperature pairs tended to be higher than those of other cultivation temperature pairs (Fig. 3). In addition, the values of H'_β/H'_γ of Rj_2Rj_3 and $Rj_2Rj_3Rj_4$ genotype soybean cultivars between low and high cultivation temperature pairs tended to be lower than the values of non-*Rj* and Rj_4 genotype soybean cultivars (Fig. 3). The values of H'_β/H'_γ of three soybean cultivars, A-250-3, B349, and C242, all of which were $Rj_2Rj_3Rj_4$ genotype soybean cultivars, tended to be comparatively lower than those of non-*Rj* and Rj_4 genotype soybean cultivars and were similar to the values of H'_β/H'_γ of Rj_2Rj_3 genotype soybean cultivars (Tables 2 and 3 and Fig. 3).

Comparison of bradyrhizobial communities using MDS analysis. The result of cluster analysis based on the Bray-Curtis similarity measure is shown in Fig. 4. The dendrogram indicated that MDS plots of combinations of cultivation temperature and soybean cultivars were classified broadly into four clusters, expressed in clusters I, II, III, and IV (Fig. 4). The result of MDS analysis is shown in Fig. 5. The MDS plot at a low cultivation temperature was classified mainly into clusters I, II, and III; the MDS plot at the middle cultivation temperature was classified mainly into clusters III and IV, and most of the MDS plot at a high cultivation temperature was classified into cluster IV (Fig. 4 and 5). Most of the MDS plots of combinations of cultivation temperature and soybean cultivar were translated to clusters from left to right in the figures (from low cluster numbers to high cluster numbers) with increasing cultivation temperature, except for Akishirome, Akisengoku, and Fukuyutaka, which did not follow this pattern partially (Fig. 5). At a high cultivation temperature, MDS plots of six soybean cultivars—Bonminori, CNS, IAC-2, Hill, A-250-3, and B349—were distributed in cluster IV, the rightmost region, and even at a low cultivation temperature, MDS plots of six soybean cultivars—Bonminori, CNS, Hardee, A-250-3, B349, and C242—were distributed in cluster III. At a low cultivation temperature, the MDS plots of five soybean cultivars—Akishirome, Bragg, Orihime, Fukuyutaka, and Hill—were distributed in cluster I, the leftmost region. On the other hand, the MDS plot of Akishirome at a high cultivation temperature deviated to the higher side of cluster II (Fig. 5).

DISCUSSION

To investigate the influence of cultivation temperatures and soybean *Rj* genotypes on the nodulation tendency and community

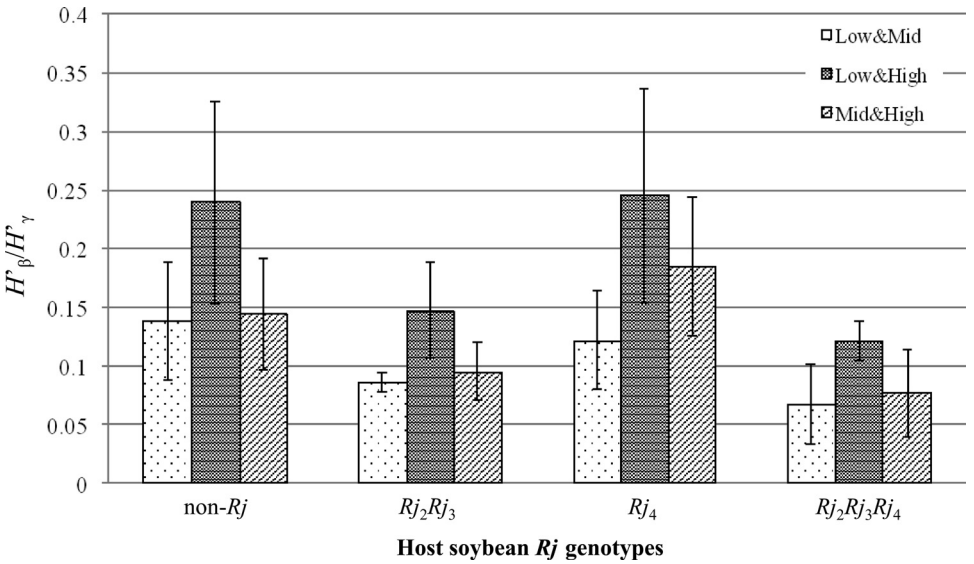

FIG 3 Difference in beta diversity compared to gamma diversity (H'_β/H'_γ) among pairs of cultivation temperatures. Each value is expressed as the mean ± the standard error ($n = 3$ or 4).

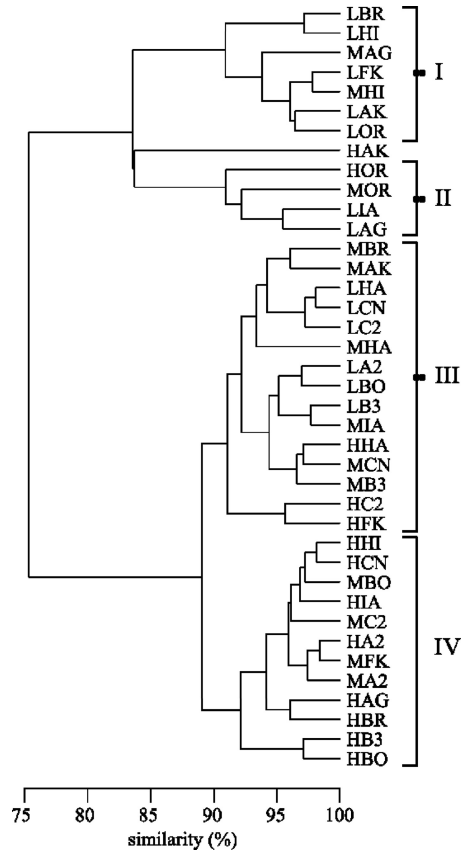

FIG 4 Dendrogram based on the Bray-Curtis similarity measure. The numbers I, II, III, and IV indicate clusters I, II, III, and IV, respectively. The names of OTUs were expressed in combinations of cultivation temperature and soybean cultivar. Low, middle, and high cultivation temperature are represented by L, M, and H, respectively. Each soybean cultivar is abbreviated as follows: AK, Akishirome; BR, Bragg; OR, Orihime; BO, Bonminori; CN, CNS; HA, Hardee; IA, IAC-2; AG, Akisengoku; FK, Fukuyutaka; HI, Hill; A2, A-250-3; B3, B349; and C2, C242.

structure of indigenous bradyrhizobia, we isolated 780 DNA samples from 13 cultivated soybean cultivars under three patterns of cultivation temperatures. *Bradyrhizobium japonicum*, *B. elkanii*, and *Sinorhizobium fredii* are known to be major soybean-nodulating rhizobia. Since the genus *Sinorhizobium* inhabits alkaline soils (20, 28), it is reasonable to assume that all isolates in the present study belong to the genus *Bradyrhizobium* and that most are *B. japonicum*, based on the results of PCR-RFLP analysis.

With increasing cultivation temperature from low to high, the occupancy rate of the Bj123 cluster decreased and the occupancy rate of the Bj110 cluster increased. Furthermore, the soybean cultivars with Rj_2Rj_3 genotypes and $Rj_2Rj_3Rj_4$ genotypes showed a higher occupancy rate of the Bj110 cluster (50–73.8%) than other *Rj* genotype soybean cultivars (Table 1), suggesting that the host soybean *Rj* genotype affected the infection of some specific bradyrhizobia. Yamakawa et al. (34) reported that $Rj_2Rj_3Rj_4$ genotype cultivars were superior to other *Rj* genotypes for inoculation with *B. japonicum* USDA 110. In the present study, we did not demonstrate the inoculum efficiency of *B. japonicum* USDA 110; however, this previous result suggested that Rj_2Rj_3 and $Rj_2Rj_3Rj_4$ genotype soybean cultivars may enhance the occupancy rate of the inoculum of *B. japonicum* USDA 110. In addition, the occupancy rate of the Bj110 cluster in $Rj_2Rj_3Rj_4$ genotypes did not show significant differences among the three cultivation temperature conditions tested (Table 1), suggesting that $Rj_2Rj_3Rj_4$ genotype soybean cultivars were unaffected by cultivation temperature changes and may enhance the inoculum efficiency such as that of *B. japonicum* USDA 110.

We also investigated the differences in bradyrhizobial communities for the pairs of cultivation temperature. The nodulation tendencies of soybean cultivars were similar for each cultivation temperature, and differences in the community structures between low and high cultivation temperatures were relatively larger than the other comparisons, although the statistical significant difference was not detected. This possible reason is that responses of soybean cultivars for cultivation temperatures on soybean-nodulating bradyrhizobial communities are different among each soybean cultivar even in same *Rj* genotypes. Therefore, analyses of soybean-nodulating rhizobial communities on not only *Rj* genotypes but also every soybean cultivars must be conducted for environmental factors affecting soybean-nodulating rhizobial community structures such as cultivation temperature in further studies. The responses of host soybean and soybean-nodulating bradyrhizobia under cultivation conditions such as a suboptimal root zone temperature were reported previously. The lowering of temperature delayed bradyrhizobia infection of soybean roots and lowered the genistein secretion from soybean roots (38, 39). It also appeared to inhibit the expression of the nodulation (*nod*) gene of soybean-nodulating bradyrhizobia (37). However, Pan and Smith (16) reported that the concentration of daidzein secreted from soybean roots increased with decreasing root zone temperature. The physiological factors of bradyrhizobia involved in the nodulation are the expression of the *nod* gene and growth capability in soil and rhizosphere. Yokoyama (36) demonstrated that the expression level of the *nod* gene of three *Bradyrhizobium* strains, *B. japonicum* USDA 110 strain, *B. elkanii* USDA 76 strain, and *Bradyrhizobium* sp. TARC 64 strain (isolates from Thailand soil [35]), which were mutants of *nodY-lacZ* fusion, were different depending on the incubation temperature (20, 23, 26, 30, 33, 35, 37, and 40°C) and suggested that the transcriptional responses of the *nod* gene of USDA 110 strain and USDA 76 strain were distinctly different at 23 to 35°C. Saeki et al. (23) demonstrated that the population occupancy of four *Bradyrhizobium* USDA strains, *B. japonicum* USDA 6^T, 38, and 123 and *B. elkanii* USDA 76^T, in soil microcosms changed with different temperature conditions and indicated that USDA 76^T was dominant over a wide range of temperature conditions, especially at higher temperature, whereas USDA 123 was dominant at low soil temperatures. These results suggested that temperature is one of the environmental factors affecting the infection of soybean and the bradyrhizobia occupancy in soils. Furthermore, Duzan et al. (5) reported that the deformations of soybean root hair decreased with decreasing root zone temperature. The infection and nodulation of soybean by bradyrhizobia under different temperature conditions may be affected by other, as-yet-unknown factors as well.

The results of MDS analysis indicated the change of bradyrhizobial communities with cultivation temperature, and cluster analysis based on the Bray-Curtis similarity measure showed that MDS plots were classified broadly into four clusters (Fig. 4 and 5). The result of MDS analysis and the number of host soybean isolates belonging to each cluster were compared, and the comparison showed that the distribution of the MDS plot was affected by

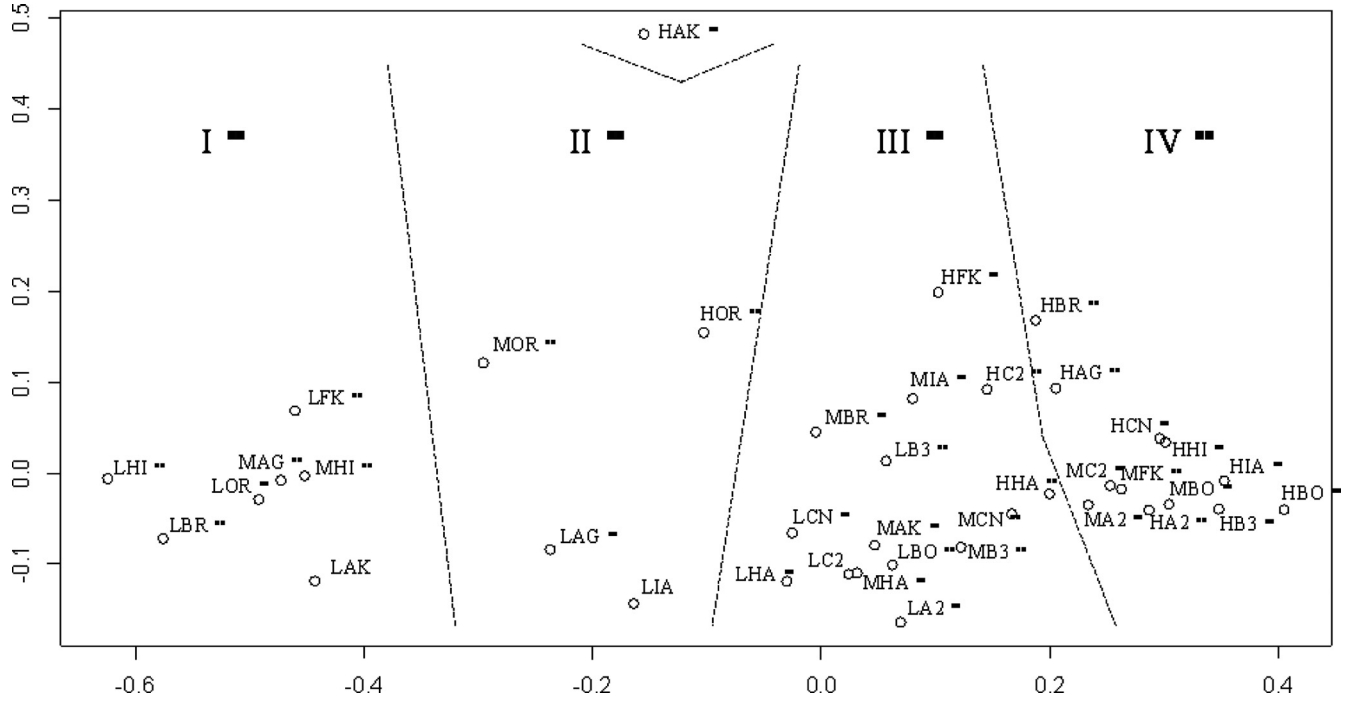

FIG 5 Multidimensional scaling (MDS) plot of bradyrhizobial communities in combinations of soybean cultivars and cultivation temperatures based on the Bray-Curtis similarity measure. The numbers I, II, III, and IV indicate clusters I, II, III, and IV, respectively. The lines show the boundaries of each cluster. The names of OTUs are expressed in combinations of cultivation temperature and soybean cultivar. The expression of cultivation temperatures and soybean cultivars refers to Fig. 4.

the two major bradyrhizobial clusters, the Bj110 cluster and the Bj123 cluster (Fig. 2 and 5). The MDS plot was distributed in cluster IV when the number of Bj110 cluster was large and in cluster I when the occupancy rate of Bj123 cluster was large (Fig. 5). However, the MDS plots of six soybean cultivars with Rj_2Rj_3 genotype (Bonminori, CNS, Hardee, A-250-3, B349, and C242) in low cultivation temperature conditions were not distributed in cluster I or II (Fig. 5) because they were infected with the occupancy rate of the Bj110 cluster even under low cultivation temperature conditions (Table 1). In addition, the MDS plots of two soybean cultivars, Akishirome and Fukuyutaka, were irregular compared to the translation of MDS plots of other soybean cultivars, but these soybean cultivars were partially translated between clusters from the left region to the right region with increasing cultivation temperature (Fig. 5). Akishirome and Fukuyutaka may have optimal temperatures at the middle cultivation temperature, which is infected with the occupancy rate of the Bj110 cluster, because the MDS plots of these soybean cultivars in the middle cultivation temperature were distributed in cluster III or IV (Fig. 5). These results suggested that the host *Rj* genotypes and cultivation temperatures affected the community structure of indigenous bradyrhizobia infecting host soybean.

The *B. japonicum* USDA 110 strain has a high nitrogen-fixing ability because it carries an uptake hydrogenase (*hup*) gene (1, 19). This strain also has nitrous oxide (N_2O) reductase activity (25, 26, 27). The N_2O that is developed by defective denitrification is a strong greenhouse gas (2, 11). The utilization of *B. japonicum* USDA 110 strains as an inoculum for soybean cultivation appears to have the potential not only to increase soybean yields but also to enhance environmental preservation by the reduction of N_2O gas. However, there are unsolved issues about the effect of the cultivation temperatures and *Rj* genotypes involved in the competition between indigenous bradyrhizobia and inocula such as the *B. japonicum* USDA 110 strain. To develop and confirm an efficient inoculation technique adapted to the climate of soybean production areas, we must consider the ecological relationship among the host soybean *Rj* genotypes and environmental factors such as cultivation temperatures.

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